KOLOREKTAL ADENOMLAR VE ADENOKARSİNOMLARDA HÜCRE SİKLUSUNU DÜZENLEYEN PROTEİNLERİN VE APOPİTOZLA İLGİLİ BELİRTEÇLERİN EKSPRESYONU

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SUMMARY

OBJECTIVE: Tumor growth is regulated by a balance between proliferation, growth arrest and cell death. In this study, we have examined the expression of p27, cyclin D1, bcl-2, and bcl-x for evaluation of their roles in colon carcinoma progression.

MATERIALS and METHODS: The levels of p27, cyclin D1, bcl-2, and bcl-x expression were examined by immunohistochemistry in transitional normal mucosa adjacent to adenomas (n=30), adenomas (n=30), transitional normal mucosa adjacent to adenocarcinomas (n=63), adenocarcinomas (n=63) and metastasis (n=16). Standard streptavidin-biotin immunperoxidase method was used for immunostaining and the stained slides were examined microscopically using semiquantitative criteria.

RESULTS: Normal mucosa expressed p27 protein and adenocarcinomas displayed a decrease in the expression of this protein. Decreased expression of p27 was associated with tumor progression (p=0.026). Cyclin D1 staining was prominent in most of the adenocarcinomas and metastasis (p=0.042). Meanwhile, we could not find any relation between p27 and cyclin D1 expression. Bcl-2 and bcl-x expression also did not show any statistically significant correlation in tumor progression.

CONCLUSION: The results of this study indicate that reduced p27 and cyclin D1 protein levels play an important role in progression of colon cancer. Bcl-2, and bcl-x expression were of no role.

Key words: Colon carcinoma, cell cycle, apoptosis

Kolorektal Adenomlar ve Adenokarsinomlarda Hücre Siklusunu Düzenleyen Proteinlerin ve Apopitozla İlgili Belirteçlerin Ekspresyonu

ÖZET

AMAÇ: Tümör gelişimi proliferasyon, büyümede durma ve hücre ölümü arasındaki denge ile düzenlenir. Bu çalışmada, kolon karsinomunun gelişimindeki rollerinin değerlendirilmesinde p27, siklin D1, bcl-2 ve bcl-x expresyonunu inceledik.

GEREÇ ve YÖNTEM: p27, siklin D1, bcl-2 ve bcl-x expresyonunun düzeyleri, adenomlara komşu normal mukozada (n=30), adenomlarda (n=30), adenokarsinomlara komşu normal mukozada (n=63), adenokarsinomlarda (n=63) ve metastazlarda (n=16) immünohistokimyasal olarak incelendi. İmmün boyama için standart streptavidin-biotin immünperoksidaz metodu kullanıldı ve boyanan slaytlar yarı kantitatif kriterler kullanarak mikroskopik olarak incelendi.

BULGULAR: Normal mukozada p27 protein ekspresyonu saptandı, adenokarsinomlar ise bu protein ekspresyonunda azalma gösterdi. p27 ekspresyonunda azalma tümör progresyonu ile ilişkili idi (p=0,026). Siklin D1 boyaması adenokarsinom ve metastazların çoğunda belirgindi (p=0,042). Bu arada, p27 ve siklin D1 ekspresyonu arasında herhangi bir ilişki saptamadık. Bcl-2 ve bcl-x ekspresyonu da tümör progresyonunda istatistiksel olarak belirgin anlamlılık göstermedi.

SONUÇ: Bu çalışmanın sonucu p27 ve siklin D1 protein düzeylerinin kolon kanserinin progresyonununda önemli rolü olduğunu göstermektedir. Bcl-2 ve bcl-x ekspresyonunun ise rolü saptanmamıştır.

Anahtar sözcükler: Kolon kanseri, hücre döngüsü, apopitoz

Carcinoma of the large bowel is one of the most common malignancies in the world and by far the most common carcinoma of the gastrointestinal tract [1]. Development of colon carcinoma is characterized by an accumulation of genetic changes in tumor cells. There are many factors that determine the rate of colon carcinoma progression.

Colorectal carcinoma development is a multistep process. The development of carcinoma from adenomatous lesions is referred to as the adenoma-carcinoma sequence. Accumulation of genetic changes in tumor suppressor genes and oncogenes has been proposed to relate to the stages of colorectal carcinogenesis.

As with other tumors alterations in cell cycle regulatory mechanisms play an important role in colorectal carcinoma development and tumor growth is regulated by a balance between proliferation, growth arrest, and cell death. Uncontrolled cell proliferation is a main hallmark of cancer. Alterations in the expression or activity of proteins that are involved in cell cycle regulation are playing important

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roles in carcinoma progression [2]. Cell cycle progression is regulated by a series of cyclins; cyclin-dependent kinases (CDKs) and regulation of these kinases are also controlled by specific CDKs inhibitors (CDKI) at different phases of the cell cycle. The D-type cyclins like cyclin D1 are important in regulating the G1 checkpoint in cell cycle. p27, a member of the Cip/Kip family is a low molecular weight CDKI, which is able to arrest cell cycle progression by complexing CDKs and their activity. p27 is essential in controlling cell proliferation in many different tissues [3].

Apoptosis is a form of cell death which is regulated at the gene level and plays a central role in neoplastic transformation. The main group of genes controlling apoptosis is the bcl family, which includes both promoters and inhibitors. Bcl-2 protein expression has been associated with shorter disease free survival in some groups of patients with tumors. Anti-apoptotic protein bcl-x seems to play a major role in colorectal tumorigenesis and progression. A shift from expression of bcl-2 to bcl-x has been demonstrated during progression of colorectal tumors, and significant bcl-x overexpression has been found in the majority of colorectal cancer patients when compared with the corresponding normal colonic tissue.

In the present study, we investigated the immunohistochemical expressions of p27, cyclin D1, bcl-2, and bcl-x in adenomas, adenocarcinomas, and metastasis and also adjacent transitional normal mucosa to find their effects on progression of colorectal carcinomas.

MATERIALS and METHODS

In the present study, the tissues obtained from a total of 30 adenomas, 63 adenocarcinomas, and 16 adenocarcinoma metastasis between the years 1998 and 2006 were examined. The hematoxylin and eosin (H&E) stained slides of each case were taken from the pathology archives and reviewed in each tumor.

Immunohistochemistry

For each case, the most representative tumor tissue block was chosen and 5 µm sections were taken to polyL lysin coated slides for immunuhistochemical staining. Standard streptavidin biotin immunperoxidase method was used for immunostaining with p27 (AM396, BioGenex, CA, USA), cyclin D1 (Novo Castra, Newcastle, UK), bcl-2 (MS123, Neomarkers, CA, USA), and bcl-x (A3535, Dako, Ely, UK) antibodies. Appropriate tissue sections known to react with both antibodies as positive controls for each primary antibody were also stained simultaneously.

Cells that had nuclear staining were considered to be positive for p27 and cyclin D1 and cytoplasmic immunostaining were accepted as positive for bcl-2 and bcl-x. The amounts of immunopositive cells were estimated semiquantitatively: grade 1+ corresponds to 10-50%, grade 2+ to 50-75%, and grade 3+ more than 75% positive cells (4).

Statistical analysis

Data were analyzed by computer software SPSS for Windows 10.0. A p value <0.05 was considered statistically significant. Stage, grade, and type of the lesion (adenoma, adenocarcinoma, metastasis, adjacent normal mucosa) were compared with immunohistochemical scores by Pearson correlation method. Paired comparisons between groups were analyzed using Spearman test.

RESULTS

The gender distribution of our cases were 62 men and 47 women. The median age of the patients was 63.5 years, with a range of 3699. The size of the resected adenocarcinomas ranged between 1 and 15 cm (mean 5.66 cm). Four (6.3%) of the adenocarcinomas were well differentiated, 46 (73%) were moderately differentiated, and 13 (20.6%) were poorly differentiated. According to the Astler Coller classification, 11 (17.5%) were staged as B1; 31 (49.2%) were B2; one (1.6%) was C1; and 20 (31.7%) were C2. The localizations of metastasis were liver in 13 and lungs in three.

Immunoreactivity for p27 was observed in 22 of the 63 adenocarcinomas, 21 of the 30 adenomas and 5 of the 16 metastasis (Figure 1). None of the adenocarcinomas and metastasis showed strong immunoreactivity. There was a significant reduction of p27 staining in adenocarcinomas and metastasis when compared to normal mucosa and adenomas ($p=0.001$). Strongly positive staining for p27 was found dominantly in transitional normal mucosa adjacent to adenocarcinomas. p27 was also correlated with bcl-x expression ($r=0.278$, $p=0.001$).

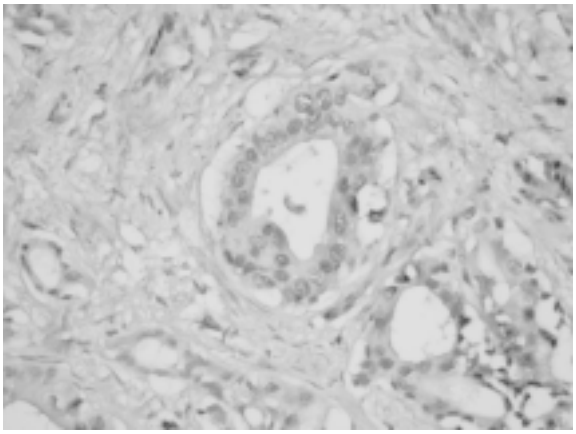

Figure 1. p27 immunohistochemical staining in colon adenocarcinoma (x400).

Cyclin D1 immunoreactivity was detected in 48 of 63 adenocarcinomas, 17 of 30 adenomas, and 10 of the 16 metastasis (Figure 2). Although there was no significant relation between cyclin D1 staining scores and tumor grade, immunoreactivity was prominent in most of the adenocarcinomas and metastasis (r=0.143, p=0.042). Cyclin D1 also showed significant correlation with bcl-2 and bcl-x (r=0.325, p=0.001 and r=0.170, p=0.024, respectively). In statistical analysis, there was also no relation between p27 and cyclin D1 expression.

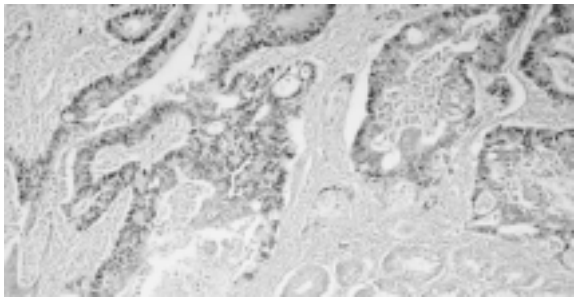

Figure 2. Immunostaining of cyclin D1 in colon adenocarcinoma (x200).

Forty-one of 63 adenocarcinomas, 14 of 30 adenomas, 5 of 16 metastasis showed positive staining with bcl-2, while 46 adenocarcinomas, 19 adenomas, 11 metastasis were positive with bcl-x. Tables 1 and 2 briefly demonstrate p values of paired comparisons and immunoreactivity of the lesions.

None of the immunohistochemical markers studied showed significant correlation with tumor grade and stage. The distribution of the immunohistochemical staining according to tumor grade and stage is shown in Table 3.

Table 1. Correlation between p27, cyclin D1, bcl-2, and bcl-x.

	Cyclin D1	Bcl-2	Bcl-x
p27	r=0.021 p=0.788	r=-0.002 p=0.977	r=0.278 p<0.001
Cyclin D1		r=0.325 p<0.001	r=0.170 p=0.026
Bcl-2			r=-0.081 p=0.284

DISCUSSION

Low p27 expression has been reported to be a poor prognostic factor in a variety of human cancers including prostate, lung, gastrointestinal tract cancers, squamous cell carcinomas and has been found to play role in the differentiation of pancreatic cancer [5-8]. Belluco demonstrated that the immunohistochemical assessment of p27 protein provides prognostic information in patients with stage II and III disease in

Table 2. Immunohistochemical staining of the lesions.

	Adenoma	Mucosa adjacent to adenoma	Carcinoma	Mucosa adjacent to carcinoma	Metastasis
p27					
N	30	30	63	63	16
None	9	6	41	42	11
+	11	13	18	11	3
++	9	9	4	6	2
+++	1	2	-	4	-
Total positive	21	24	22	21	5
Cyclin D1					
N	30	30	63	63	16
None	13	14	15	25	6
+	6	7	12	9	3
++	8	8	25	27	6
+++	3	1	11	2	1
Total positive	17	16	48	38	10
Bcl-2					
N	30	30	63	63	16
None	16	13	22	20	11
+	5	9	23	17	2
++	7	7	16	23	3
+++	2	1	2	3	-
Total positive	14	17	41	43	5
Bcl-x					
N	30	30	63	63	16
None	11	9	17	22	5
+	10	9	19	24	9
++	9	12	24	17	2
+++	-	-	3	-	-
Total positive	19	21	46	41	11

Table 3. Distribution of immunohistochemical staining according to the tumor grade and stage.

		p27		Cyclin D1		Bcl-2		Bcl-x	
		+	-	+	-	+	-	+	-
Grade									
	Well	2	2	4	-	3	1	3	1
	Moderate	16	30	35	11	30	16	35	11
	Poor	4	9	9	4	8	5	8	5
Stage									
	A	-	-	-	-	-	-	-	-
	B1	6	5	9	2	7	4	9	2
	B2	10	21	23	8	20	11	21	10
	C1	-	1	1	-	1	-	-	1
	C2	6	14	15	5	13	7	16	4

colorectal adenocarcinomas [9]. The absence of p27 protein was independently associated with a shorter relapse-free survival, overall survival and was inversely correlated with tumor stage. Decreased p27 expression has been associated with large size tumor, positive lymph nodes, and with poor survival. Loss of p27 could be one of the factors responsible for the development of metastases [10,11]. Tornillo et al found out that p27 expression was more frequent in left sided tumors [10]. It is evident that deregulation of p27 has a profound effect on tumor progression and is found to be an accurate and independent prognostic marker [12,13]. Although some authors recorded that >90% of colorectal tumors expressed p27, in the present study, we found positive staining in 34% of adenocarcinoma cases and the staining was weaker than adenomas indicating that p27 is down-regulated during the neoplastic process. These results confirmed the previous reports (12). According to the results of our series, low expression of p27 was independent with the stage of the disease; so its low expression or absence may help us to identify aggressive tumor behavior in localized disease.

Abnormal expression of cyclin D1 and cyclin E have also been reported in a variety of malignancies [3,14,15]. Although some authors found that cyclin D1 failed to have prognostic significance [16-18], it was mostly found to be overexpressed in colorectal carcinomas [3,19,20]. Moreover, it was overexpressed in the lymph node metastasis at a higher frequency than the primary lesion and so it was thought that this protein might suggest a role in the metastatic process [21]. Immunohistochemical analysis of our study showed cyclin D1 expression in 76.2% of adenocarcinomas which is similar to previous studies [22]. This cyclin D1 expression may be related with colorectal carcinogenesis. However, there was no statistically significant association between cyclin D1 expression and tumor grade. Kristt also could not find any relation with grade and concluded that increased cyclin D1 may be an early event in colorectal carcinogenesis and only cyclin D1 overexpression is not likely to predict biological behavior [19,23]. Holland found that cyclin D1 was associated with tumor differentiation and noted that patients expressing cyclin D1 had improved survival [4]. Overexpression of cyclin D1 might have more important role in colon cancer progression and other cyclins may be more important in tumor differentiation. Cyclin D1 is inducible by a variety of oncogene products. Multiple independent mechanisms of cell cycle deregulation may be present during colonic carcinogenesis, whereas other studies have failed to find any correlation between cyclin D1 and cyclin E overexpression and the clinicopathologic factors of colorectal cancer [20].

In some studies there was an association between p27 and cyclin D1 expression indicating that the balance between the two opposing regulators was important for the end result of cell cycle progression. We could not find any relation between p27 and cyclin D1 expression, as reported in some other studies [24]. There is compelling evidence in the literature for p27 to be regarded as a tumor marker and this must be examined in the light of its interactions with other proteins, most notably cyclin E [13,25,26]. In addition, p21 protein expression was correlated with the presence of cyclin D1 suggesting a possible co-regulatory control mechanism for these proteins [22].

Dysregulation of apoptosis is prominent in colorectal carcinogenesis. A shift from expression of bcl-2 to bcl-x has been demonstrated during progression of colorectal tumors, and significant bcl-x overexpression has been found in the majority of colorectal cancer patients when compared with the corresponding normal colonic tissue. These results suggest that expression of both molecules might be a useful prognostic marker in colorectal cancer [27]. Abnormal bcl-2 immunoreactivity has been observed in early dysplastic and neoplastic lesions of the colon and small intestine, suggesting that bcl-2 alterations occur early in the sequence of molecular events, leading to gastrointestinal neoplasia [28]. Grizzle hypothesized that the prognostic importance of bcl-2 expression may be limited to spesific subgroups of patients as well as anatomic location of the tumor [29]. Although, several studies demonstrated that altered

expression of bcl-2 protein (or apoptotic markers) or high expression of bcl-2 and low expression of bax could be expected in some colonic cancers [30,31], bcl-2 and bcl-x expressions were not associated with progression of colon carcinoma in the present study. Their expression did not show any significant correlation between various clinicopathological parameters. Not only our study but also Paradiso's could not find clinically relevant associations between markers controlling apoptosis and clinicopathological parameters [32]. Bukholm also found that bcl-2 and bax were not of prognostic value [18].

One of the major goals of cancer therapy in general is induction of apoptosis (cell death) in the malignant cells. More targeted treatment modalities such as gene therapy also hope to induce apoptosis. p27 along with other apoptosis-regulating genes such as p53, p21, E2F, PTEN, and Caspase, are all potential targets for more specific, directed therapeutic regimes. Several clinical trials are underway in which the tumor-suppressor and apoptosis-inducing properties of genes are being used in new and novel treatment protocols [13].

The results of this study indicate that reduced p27 protein levels and cyclin D1 overexpression play an important role in progression of colon cancer. We did not find a clear association between expression of apoptosis related markers and prognostic features or carcinoma progression. Controlling the activation and progression of the apoptotic programme is a complex cascade. Progress may depend on the participation of other regulatory signals.

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